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Bullera arundinariae sp. nov., A New Species of Ballistoconidium-forming Yeast, Isolated from a Plant in Thailand

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A ballistoconidium-forming yeast strain was isolated from a leaf collected in a protected rain forest in Thailand. It was found to represent a new anamorphic species in the Hymenomycetes, based on analysis of the nucleotide sequences of small subunit (SSU) rDNA, and internal transcribed spacer regions, including 5.8S rDNA and D1/D2 domain of large subunit (LSU) rDNA, and it was described as *Bullera arundinariae* Fungsin, Takashima & Nakase sp. nov. In a phylogenetic tree based on SSU and D1/D2 domain of LSU rDNA sequences, it is located at a position separated from clusters that include the remaining hymenomycetous ballistoconidium-forming yeasts and related taxa.

Key words : *Bullera arundinariae* sp. nov., a new ballistoconidium-forming yeast, Thailand, phylloplane yeast

INTRODUCTION

The genus *Bullera* Derx, a ballistoconidium-forming anamorphic yeast genus, is characterized by the formation of symmetrical ballistoconidia, the presence of xylose in whole-cell hydrolysates, a lack of stalked-conidia, and Q-10 as the major ubiquinone isoprenologue (1). Yeasts of *Bullera* are widely distributed in the phyllosphere of Thailand (10). Takashima and Nakase (14) found that a yeast strain, isolated from a plant collected in the southeast seacoast of Bangkok, represented a new species of this genus, and described it as *Bullera penniseticola*. This is a first record of a new species of *Bullera* in Thailand.

In the course of a survey of ballistoconidium-forming yeasts in a tropical protected rain forest in northeastern Thailand, we isolated a *Bullera* strain that cannot be assigned to any known species. This yeast is the second new species of *Bullera* found in Thailand, and it is herein described as *Bullera arun-*

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dinariae sp. nov.

MATERIALS AND METHODS

Isolation of yeast strain employed. Strain TY-146, employed in the present study, was isolated from a leaf of *Arundinaria pusilla* that Cheval & A. Camus (Gramineae) collected in a protected rain forest in Sakaerat, Nakhon Rachasima Province, Thailand in Nov. 1996. It was isolated by the ballistoconidia-fall method previously reported (9), with YM agar (Difco Lab., Detroit, USA), without any antibacterial or antifungal agents. Immediately after purification by conventional streaking technique, the isolate was preserved at −80 ℃, suspended in YM broth supplemented with 10 % (w/v) glycerol.

Examination of morphological, physiological, and biochemical characteristics

Most of the morphological, physiological, and biochemical characteristics were examined according to Yarrow (18). The assimilation of nitrogen compounds was examined on solid media using starved inoculums, according to Nakase and Suzuki (7). Vitamin requirements were determined as described by Komagata and Nakase (5). The maximum growth temperature was determined in YM broth (Difco Lab., Detroit, USA) using metal block baths.

Ubiquinone systems

Cells were cultivated in 500 m*l* Erlenmeyer flasks containing 250 m*l* of YM broth on a rotary shaker at 150 rpm at 25 ℃, and were harvested in the early stationary growth phase and washed with distilled water. Extraction, purification, and identification of ubiquinones were carried out according to Nakase and Suzuki (8).

Xylose in the cells

Cells were cultivated in 500 m*l* Erlenmeyer flasks containing 250 m*l* of YM broth on a rotary shaker at 150 rpm at 25 ℃, and were harvested in the early stationary phase. The cells were washed with distilled water and dried with acetone. Xylose in the cells was analyzed according to the method reported by Suzuki and Nakase (13).

Isolation and purification of nuclear DNA

Cells were grown in 500 m*l* Erlenmeyer flasks containing 250 m*l* of YM broth on a rotary shaker at 150 rpm at 25 ℃, and were harvested in the logarithmic growth phase. The cells were washed with distilled water and freeze-dried. Isolation and purification of nuclear DNA were done according to Takashima and Nakase (15).

DNA base composition

The DNA base composition was determined by HPLC after enzymatic digestion of DNA to deoxyribonucleosides as described by Tamaoka and Komagata (16). A DNA-GC Kit (Yamasa Shoyu Co., Ltd., Chiba, Japan) was used as the quantitative standard.

Sequencing and phylogenetic analysis

The nucleotide sequences of SSU (18S) rDNA, and the internal transcribed spacer regions (ITS 1 and 2), including 5.8S rDNA, were directly determined using PCR products according to Sugita and Nakase (12). The nucleotides sequences of D1/D2 domain of LSU (26S) rRNA gene were directly determined using PCR products according to Kurztman and Robnett (6) and Fell et al. (2). The fragment was amplified with forward primer 934F (5′-CTGCGAAAGCATTTGCCAAGG-3′) and reverse primer LR6 (5′-CGCCAGTTCTGCTTACC-3′). Amplification was performed for 30 cycles with denaturation at 94 ℃ for 1 min, annealing at 52 ℃ for 1 min and 30 sec, and extension at 72 ℃ for 2 min and 30 sec. The amplified DNA was purified with the QIA quick PCR purification kit (Qiagen, Hilden, Germany). Cycle sequencing of the D1/D2 domain was performed with forward primer F63 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and reverse primer LR3 (5′-GGTCCGTGTTTCAAGACGG-3′), using an ABI BigDye cycle sequencing kit, and electrophoresis was done on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Stafford, USA)

The sequences of SSU rDNA, ITS regions, including 5.8S rDNA and D1/D2 domain, determined in this study were deposited at the GenBank database under

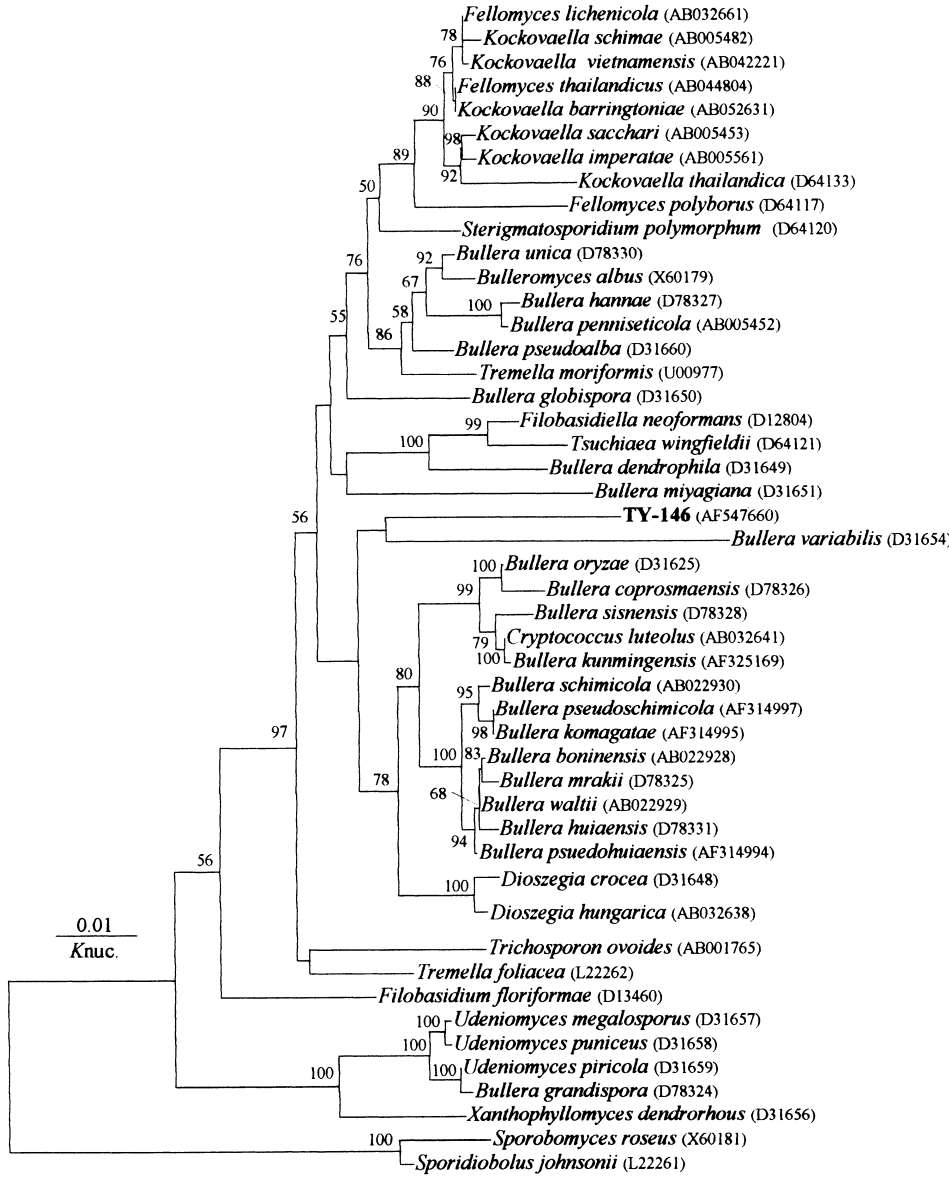

Fig. 1. Phylogenetic tree of strain TY-146 and related taxa based on the SSU rDNA sequences

The tree was constructed from evolutionary distance data according to Kimura (4), using the NJ method (11) with bootstrapping (3). The numerals given at nodes represent results from 1,000 replicated bootstrap samplings ; a frequency of less than 50 % is not indicated.

the following accession numbers : SSU rDNA, AF547660 ; ITS region including 5.8 rDNA, AF547662 ; D1/D2 domain of LSU rDNA, AF547661. Reference sequences used for the phylogenetic study, shown in Fig. 1, were retrieved from the database. Generated sequences were aligned with related species in hymenomycetous yeasts using the CLUSTAL W ver. 1.74 computer program (17). The phylogenetic tree was constructed from evolutionary distance data according to Kimura (4), using the neighbor-joining (NJ) method (11), in the PHYLIP computer program. Sites where any gaps existed in any sequences were excluded. Bootstrap analyses (3) were performed from 1,000

random resamplings.

RESULTS AND DISCUSSION

Strain TY-146 formed rotationally symmetrical ballistoconidia ; it did not produce stalked conidia ; had Q-10 as the major ubiquinone isoprenoloque, and it had xylose in the cells. These characteristics coincided well with those of the genus *Bullera* (1). Therefore, this strain was assigned to this genus.

The SSU rDNA sequence of TY-146 was determined in the present study. A phylogenetic tree was constructed based on the SSU rDNA sequences of this strain and the related species by the NJ method (Fig. 1). In this tree, TY-146 is located at a position distant from the known species of the genus *Bullera* and related taxa, showing a similarity value of less than 93 %. The correct phylogenetic position of TY-146 is not clear, since the bootstrap value is not high in this tree.

The ITS region (including 5.8S rDNA) sequence of the strain TY-146 was also determined. The total lengths of ITS 1-5.8S rDNA-ITS2 region of this strain is 483 nucleotides. The lengths of its ITS1 and ITS2 regions are 134 and 203 nucleotides, respectively. From the result obtained from BLAST analysis in the ITS region, the most closely related species was *Sirobasidium intermedium* CBS 7805. However, the nucleotide differences in their ITS1 and ITS2 regions overall between the two strains are high, at 85 nucleotides (28.2 %). Likewise, that value between TY-146 and *Bullera variabilis*, the most closely related species in the 18S rDNA phylogenetic tree, is high, at 77 nucleotides (35.8 %).

In addition to these, the sequence of the D1/D2 domain of LSU rDNA of TY-146 was determined, and a phylogenetic tree was constructed based on the alignment of D1/D2 sequences of this strain with related species of *Bullera* and other hymenomycetous yeast taxa (Fig. 2). The tree showed that TY-146 is located at a position distant from known species of the genus *Bullera* and other hymenomyceotous yeasts. The nucleotide differences of the D1/D2 domain between TY-146 and the most closely related strain, *Cryptococcus* sp. KCTC 17077, were 60 nucleotides (12 %). Fell et al. (2) reported that strains that differed by two or more nucleotides in the D1/D2 domain represented different taxa. Based on this guideline mentioned above, we have concluded that TY-146 represents a new species of the genus *Bullera*. Further study is required to presume the correct phylogenetic position of this species. A description of a new species is provided below.

Description of *Bullera arundinariae* Fungsin, Takashima & Nakase, sp. nov.

In liquido "YM," post dies 3 ad 25 ℃, cellulae ovoideae, ellipsoideae, aut longi-ellipsoideae, 2.0-3.8 × 3.7-7.5 μm, Sedimentum formatur. In agaro "YM", post unum mensem ad 17 ℃, cultura laetocremea, nitida, butyracea, margine glabra. Mycelium et pseudomycelium non formantur. Ballistosporae globosae aut subglobosae, 3.8-5.0 × 6.3-7.5 μm. Fermentatio nulla. Glucosum, galactosum, sucrosum, maltosum, cellobiosum, trehalosum, lactosum (lente et exiguum), melibiosum, raffinosum, melezitosum, amylum solubile, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, erythritolum, ribitolum, galactitolum, D-mannitolum, D-glucitolum, α-methyl-D-glucosidum, salicinum, glucono-δ-lactonum (lente), acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum D-glucuronicum, acidum D-galacturonicum, acidum succinicum, acidum citricum et inositolum assimilantur at non L-sorbosum, inulinum, ethanolum, glycerolum nec acidum DL-lacticum. Amonium sulfatum et L-lysinum assimilantur at non kalium nitricum, natrium nitrosum, cadaverinum nec ethylaminum. Maximum temperatura crescentiae : 29 -30 ℃. Ad crescentiam thiaminae necessarium est. Diazonium caeruleum B positivum. Proportio molaris guanini + cytosini in acido deoxyribonucleico : 58.7 mol % per HPLC. Ubiquinonum majus : Q-10. Teleomorphosis ignota.

Holotypus : Stirps TY-146, isolatus ex folio *Arundinariae pusillae* Cheval & A. Camus, in pluvial sylva tropica, Nakorn Rachasima Province in Thailandia ; cultura viva ex holotypo huius speciei conservatur in collectionibus culturarum in 'Thailand Institute of Scientific and Technological Research (TISTR)', Chatuchak, Bangkok, Thailandia ut

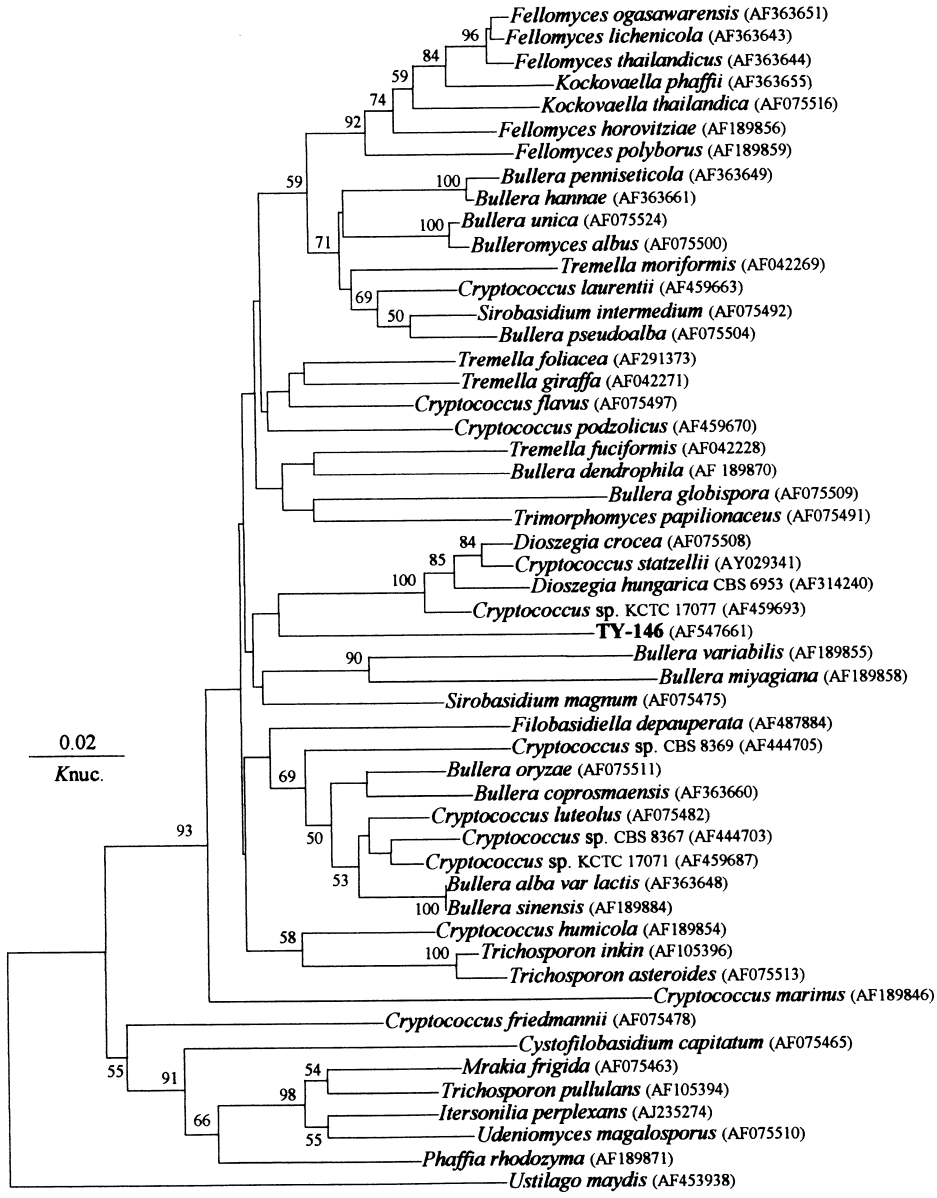

Fig. 2. Phylogenetic tree of strain TY-146 and related taxa based on the D1/D2 domain of LSU rDNA sequences

The tree was constructed from evolutionary distance data according to Kimura (4), using the NJ method (11) with bootstrapping (3). The numerals given at nodes represent results from 1,000 replicated bootstrap samplings ; a frequency of less than 50 % is not indicated.

TISTR 5798 in statu lyophilo, item in collectionibus culturarum ut JCM 11818 in statu lyophilo quas 'Japan Collection of Microorganisms (JCM)', Wako, Saitama, Japonia sustentat.

Growth in YM broth : After 3 days at 25 ℃, cells are ovoidal, ellipsoidal, or long ellipsoidal, 2.0-3.8 × 3.7-7.5 μm (Fig. 3A). After 1 month at 17 ℃, a sediment is formed.

Growth on YM agar : After 1 month at 17 ℃, a streak culture is light cream-colored, smooth , soft, shinny, wet and mucoidal, and it has an entire margin.

Dalmau plate culture on corn meal agar :

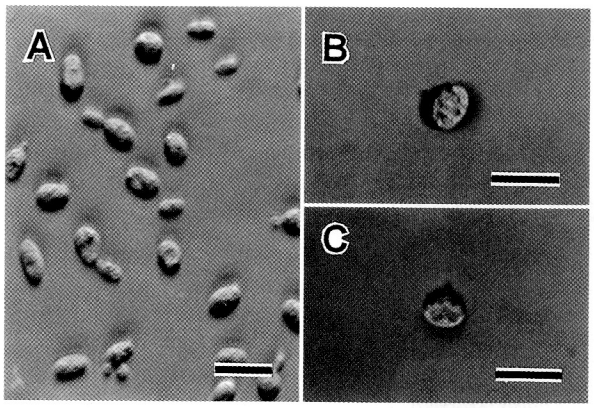

Fig. 3. Morphology of *Bullera arundinariae*

A) Vegetative cells of *B. arundinariae* TY-146 grown in YM broth for 3 days at 25 ℃.

B, C) Ballistoconidia of *B. arundinariae* TY-146 produced on corn meal agar after 7 days at 25 ℃.

Scale bars indicate 10 μm.

Mycelium and pseudomycelium are not formed.

Production of ballistoconidia : Ballistoconidia are rarely produced on corn meal agar. They are globose or subglobose, 3.8-5.0 × 6.3-7.5 μm (Fig. 3B, C).

Fermentation : Absent

Assimilation of carbon compounds :

Glucose	+	Ethanol	−
Galactose	+	Glycerol	−
L-Sorbose	−	Erythritol	+
Sucrose	+	Ribitol	+
Maltose	+	Galactitol	+
Cellobiose	+	D-Mannitol	+
Trehalose	+	D-Glucitol	+
Lactose	+ (latent & weak)	α-Methyl-D-glucoside	+
Melibiose	+	Salicin	+
Raffinose	+	Glucono-δ-lactone	+ (latent)
Melezitose	+	2-ketogluconic acid	+
Inulin	−	5-ketogluconic acid	+
Soluble starch	+	DL-Lactic acid	−
D-Xylose	+	D-Glucuronic acid	+
L-Arabinose	+	D-Galacturonic acid	+
D-Arabinose	+	Succinic acid	+
D-Ribose	+	Citric acid	+
L-Rhamnose	+	Inositol	+

Assimilation of nitrogen compounds :

Ammonium sulfate	+	Ethylamine hydrochloride	−
Potassium nitrate	−	L-Lysine hydrochloride	+
Sodium nitrite	−	Cadaverine dihydrochloride	−

Maximum growth temperature : 29-30 ℃

Vitamin required for growth : Thiamine

Production of starch-like substances : Negative

Growth on 50 % (w/w) glucose yeast extract agar : Negative

Urease : Positive

Liquefaction of gelatin : Negative

Diazonium Blue B reaction : Positive

G + C content of nuclear DNA : 58.7 mol % (by HPLC)

Major ubiquinone : Q-10

Xylose in the whole cell hydrolysate : Present

Holotype : Strain TY-146, isolated by B. Fungsin, M. Takashima, and T. Nakase. A living culture of exholotype of this species is deposited in the culture collection of the Thailand Institute of Scientific and Technological Research, Bangkok, Thailand, as TISTR 5798, in a lyophilized state, and also in the culture collection as JCM 11818, in a lyophilized state, that the Japan Collection of Microorganisms, Wako, Saitama, Japan, maintains.

Etymology : The specific epithet of this species is derived from the generic name *Arundinaria* of the host plant from which this species was isolated.

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タイ産の植物から分離した射出胞子形成酵母の新種，*Bullera arundinariae* sp. nov.

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タイの保護熱帯雨林で採集した植物葉から射出胞子形成酵母1株を分離した．Small subunit（SSU）rDNA，5.8S rDNAを含むInternal transcribed spacer領域，およびLarge subunit（LSU）rDNAのD1/D2領域の塩基配列の解析などから，分離株は菌蕈綱（Hymenomycetes）に属するアナモルフ酵母の新種であることが明らかになったので，*Bullera arundinariae* Fungsin，Takashima & Nakaseと命名した．SSU rDNAおよびLSU rDNAのD1/D2領域の塩基配列に基づく分子系統樹では，本種は菌蕈綱に含まれるほかの射出胞子形成酵母および関連酵母分類群とは離れた位置にあった．